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Red squirrels in the British Isles are infected with leprosy bacilli

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Abstract: Leprosy, caused by infection with *Mycobacterium leprae* or the recently discovered *Mycobacterium lepromatosis*, was once endemic in humans in the British Isles. UK red squirrels (*Sciurus vulgaris*) have increasingly been observed with leprosy-like lesions on the head and limbs. Using genomics, histopathology and serology we found *M. lepromatosis* in squirrels from England, Ireland and Scotland, and *M. leprae* in squirrels from Brownsea Island, England. Infection was detected in overtly diseased and seemingly healthy animals. Phylogenetic comparisons of British and Irish *M. lepromatosis* with two Mexican strains from humans showed they diverged from a common ancestor around 27,000 years ago whereas the *M. leprae* strain is closest to one that circulated in Medieval England. Red squirrels are thus a reservoir for leprosy in the British Isles.

One Sentence Summary: Diseased British and Irish red squirrels are infected with two different bacteria that cause leprosy in humans and represent a potential zoonotic threat.

48 **Main text - 1827 words**

49 **Main text, legends, etc. ~ 2355 words without references**

50

51 **Main Text:** Often considered a disease of the past, leprosy remains a public health problem
52 in certain low and middle-income countries with ~220,000 new cases reported annually (1).
53 Leprosy was rife in Europe in the Middle Ages but disappeared during the 15th-16th centuries
54 probably because of social segregation, other infectious diseases such as plague or changes in
55 host immunity (2–5). Today, all British clinical cases occur in individuals with a history of
56 residence in a leprosy endemic country (6). The disease manifests in different forms, ranging
57 from multibacillary, or lepromatous, to paucibacillary, or tuberculoid, depending on the
58 immunogenetics of the host (4). In all forms, skin lesions are accompanied by peripheral nerve
59 damage, which causes sensory loss and may lead to deformities.

60 It was generally accepted that leprosy resulted solely from inter-human transmission
61 of *M. leprae* but in recent years compelling evidence emerged from the southern USA for
62 zoonotic cases following exposure to infected nine-banded armadillos (*Dasypus novemcinctus*)
63 (7–9). Furthermore, *M. leprae* was considered to be the sole causative agent of leprosy until
64 2008 when a new species, *M. lepromatosis*, was identified in patients with diffuse lepromatous
65 leprosy (DLL) (10). Such cases were primarily associated with Mexico and the Caribbean
66 region (11). Comparison of the genome sequences of *M. lepromatosis* and *M. leprae* revealed
67 that despite separating millions of years ago, the two genomes are remarkably similar in their
68 size, organization and (pseudo)gene content, but show only 88% sequence identity (11).

69 The Eurasian red squirrel *Sciurus vulgaris* is a widespread Palearctic species found
70 from Ireland in the West to Kamchatka in the East (12, 13). However, in the United Kingdom
71 (UK) the *S. vulgaris* population of ~140,000 is severely threatened by habitat loss, squirrel
72 poxvirus infection and competition with >2.5 million grey squirrels, *Sciurus carolinensis*,
73 introduced from North America (14, 15). Due to their endangered status, red squirrels are now

protected (16). Recent detection of mycobacterial infection in red squirrels was reported in Scotland, with lesions and histopathology characteristic of DLL and evidence for *M. lepromatosis* being the etiological agent (17). Similarly affected squirrels were observed on the Isle of Wight and Brownsea Island in Southern England (18) and observations of squirrel leprosy in Scotland are increasing (Fig. 1). Here, we investigated these cases using 70 red squirrel cadavers from the UK, with or without disease signs, 40 cadavers from Ireland, where no sightings of squirrels with leprosy signs have been reported, and four Scottish grey squirrel cadavers.

A differential PCR screen was implemented to detect *M. leprae* and *M. lepromatosis* DNA (11). A total of 172 tissue samples from 13 animals with and 101 without leprosy features were analyzed (tables S1, S2, (19)). Six Scottish squirrels (two without clinical signs (17)), two from Ireland (no clinical signs), and one from the Isle of Wight, England, (18) contained *M. lepromatosis*, in several tissue samples from different anatomical sites, whereas all 25 red squirrels (17 without clinical signs) tested from Brownsea Island were infected with *M. leprae* (Fig. 1, table S3). No cases of co-infection were observed (table S3). From the combined results, we concluded that 21% (21/101; 95%CI 13-30%) of the squirrels without clinical signs and all of the animals with clinical signs (13/13) harbored leprosy bacilli.

Serological tests were performed on nine diseased and 14 healthy red squirrels from Scotland and England, and the four grey squirrels. The greys were all sero-negative whereas 13/23 blood samples from red squirrels contained antibodies for the leprosy-specific antigen, phenolic glycolipid-1 (20) (table S4, (19)). Serology is useful to confirm the disease and predict infection in live animals but cannot be used for species identification as both *M. leprae* and *M. lepromatosis* produce this cell wall antigen (11).

Diseased Scottish squirrels, infected with *M. lepromatosis*, displayed a range of macroscopic lesions including alopecia, extensive swelling of the snout, lips, eyelids, the ear pinnae and limb extremities (Figs. 1, 2A, S1, tables S2, S5, (19)). Histopathological examination of four such squirrels (Fig. 2B) revealed granulomatous dermatitis, sheets of epithelioid macrophages and large numbers of acid-fast bacilli (AFB). There was neural

involvement with the presence of AFB in nerve endings; neuritis was patchy and more frequently perineural (Fig. 2C). Inflammation was not focused exclusively around nerves and was mostly dermal. There were no signs of vasculitis, but AFB were present intravascularly (Fig. 2C). Similar lesions were observed in eight squirrels from Brownsea Island infected with *M. leprae*, although these animals also harbored numerous AFB in the spleen (Fig. 2C). Overall, the macroscopic signs and histopathology were characteristic of lepromatous leprosy (Figs. 2A, B, Figs. S2, S3). From post-mortem inspection of diseased squirrels it was not possible to distinguish between infection with *M. lepromatosis* or *M. leprae*, as in human leprosy (11, 21, 22).

To obtain deeper insight into the strains responsible and to perform phylogenetic analyses we used a variety of DNA enrichment techniques (table S6) prior to Illumina sequencing since neither *M. leprae* nor *M. lepromatosis* can be cultured (19). Sufficient sequence coverage of *M. lepromatosis* genomes from seven squirrels was obtained (table S7). In parallel, we sequenced an additional genome of *M. lepromatosis*, Pl-02, from a PGL-1-seropositive patient from Sinaloa, Mexico (tables S1, S4). The resultant sequence reads were mapped against the reference *M. lepromatosis* genome sequence from a patient from Monterrey, Mexico (11) to identify polymorphisms. Consistent with previous *M. leprae* genome comparisons (9, 11, 23), there was an exceptionally high level of sequence conservation between *M. lepromatosis* strains (99.99% identity) despite their different geographic origins. The two Mexican patient isolates differed by only seven single nucleotide polymorphisms (SNPs) whereas the number of SNPs in the six British and Irish strains ranged from one to 17 on pairwise comparisons (table S8). Overall, there are roughly 400 SNPs that distinguish *M. lepromatosis* strains from Mexico and the British Isles (table S8). Clustering of Mexican and British *M. lepromatosis* strains into two distinct lineages was supported by maximum parsimony (Fig. S4) and neighbor joining (Fig. S5) phylogenetic reconstructions. Based on the *M. leprae* mutation rate (19) and using the Bayesian inference software, BEAST (24), we estimated that the British Isles and Mexican strains diverged from their most recent common ancestor around 27,000 years ago whereas the Irish and UK strains diverged as

recently as 200 years ago (Fig. 3A). The latter estimate is consistent with the date of the first campaign to reintroduce the red squirrel into Ireland from England between 1820 – 1856, following its extinction in the 17th century (12, 25). This suggests that these animals may already have been infected with *M. lepromatosis* when they were reintroduced.

Finding *M. leprae* in red squirrels in the UK was unexpected, since leprosy was eradicated from the British Isles several centuries ago, thus demonstrating that a pathogen can persist in the environment long after its clearance from the human reservoir. Furthermore, this is only the second report of *M. leprae* in non-primate species. From Bayesian and maximum parsimony analysis (Fig. 3B, fig. S4A) we note that the two closest relatives to the strain of *M. leprae* found on Brownsea Island were both from medieval Europe. Intriguingly, one of these (SK2) originated from the skeletal remains of a leprosy victim buried about 730 years ago in Winchester, a city situated a mere 70 km from Brownsea Island (Fig. 1). Like SK2, the Brownsea Island strain of *M. leprae* belongs to sequence type 3I, which forms a distinct *M. leprae* branch (Fig. 3B) (3) and is now endemic in wild armadillos in the Southern USA (9). Thus, *M. leprae* with this particular sequence type is capable of infecting at least three different hosts: humans, red squirrels and armadillos.

Since there were no obvious genomic polymorphisms restricted to the *M. leprae* 3I type that might account for this broad host range (tables S9, S10) we explored the possibility that these three species might share a major susceptibility gene and focused on *TLR1*. This candidate gene, encoding the surface-exposed Toll-like receptor 1 (TLR1) displayed on various epithelial and immune cells, is known to be associated with susceptibility to leprosy (Fig. 4A). A dysfunctional *TLR1* allele encoding an I602S variant with an altered transmembrane domain is prevalent in Caucasians and is associated with a decreased risk for leprosy (5, 26). By contrast, the *TLR1* N248S variant is associated with an increased risk of leprosy in humans. This mutation is located in the ninth repeat of the extracellular leucine-rich repeat (LRR) region of TLR1 (27). Furthermore, in nine-banded armadillos an R627G change in TLR1 (close to the Toll/Interleukin receptor (TIR) domain, Fig. 4A), seemingly confers resistance to leprosy (28). Using PCR the coding exon of *TLR1* was amplified and sequenced

from 58 red (with or without lesions) and three grey squirrels (tables S11, S12, S14 (19)). On comparison of the sequences and TLR1 alignments (table S13) no polymorphisms were observed at the same sites associated with leprosy in humans and armadillos. However, in some red squirrels, two distinct polymorphic sites exist: a single SNP leading to a S494N mutation in the nineteenth repeat of the LRR region and a cluster of linked mutations that produce S657N, L660V and N662C variants in helix 1 of the TIR domain (Fig. 4B). These mutations were found less frequently in squirrels infected with leprosy bacilli compared to healthy animals suggesting that they may confer protection (OR: 5.77, 95% CI: 1.42 - 23.41, $p=0.01$ for 494N and OR: 4.89, 95% CI: 0.98 - 24.53, $p=0.05$ for 657N-660V-662C).

It is unclear whether leprosy is contributing to the demise of the red squirrel population or how these animals became infected with *M. lepromatosis* or *M. leprae*. Since *M. lepromatosis* has only recently been discovered as a human pathogen (10), and there are few detailed case reports (10, 11, 21, 29), further investigation is required to establish its relative prevalence in wildlife compared to humans. *M. leprae* was long considered to be an obligate human pathogen that was introduced to the Americas by European settlers, prior to anthroponotic infection of armadillos, since there are no human skeletal remains with signs of leprosy from the pre-Columbian era (9). The discovery that the strain of *M. leprae* in red squirrels on Brownsea Island today is essentially the same as one that circulated in medieval England and Denmark, and highly related to the extant North American armadillo strain, raises the possibility of a second anthroponotic introduction in Europe. If this were the case, it must have occurred several centuries ago as leprosy became increasingly scarce in the British Isles after the 17th century (3). It is also conceivable that humans may have been infected through contact with red squirrels bearing *M. leprae* as these animals were prized for their fur and meat in former times (30). Our findings demonstrate that further surveys of animal reservoirs of leprosy bacilli are warranted, since zoonotic infection from such reservoirs may contribute to the inexplicably stubborn plateau in the incidence of the human leprosy epidemic despite effective and widespread treatment with multidrug therapy (1).

186 **References and Notes**

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sequence read files were deposited in Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under accession no. SRR3672737 to SRR3672758 (NCBI BioProject PRJNA325727), SRR3674396 to SRR3674450 (NCBI BioProject PRJNA325827), SRR3674451 to SRR3674453 (NCBI BioProject PRJNA325856) and SRR3673933 and representative TLR1 sequences at GenBank under accession numbers KX388139, KX388140 and KX388141. Phylogenetic trees and SNP alignments were deposited at Treebase under Study Accession URL <http://purl.org/phylo/treebase/phyloids/study/TB2:S19692>. This work was supported by grants from the Fondation Raoul Follereau, the Swiss National Science Foundation (Grant number IZRJZ3_164174) to S.T.C., the Scottish Government Rural and Environment Science and Analytical Services Division to K.S., and the Thomas O’Hanlon Memorial Award in Veterinary Medicine to F.McD.

Fig. 1. Squirrel sampling sites in the British Isles. Pie charts indicate the location of sites where squirrels were sighted or found and color-coded as indicated in the box, numbers within circles indicate different animals tested where $N > 1$. Boxed circles refer to squirrels of unknown location: I, Ireland; S, Scotland. A, Isle of Arran; B, Brownsea Island; W, Isle of Wight. The figure was drawn in R (v3.2.23 © 2015 The R Foundation for Statistical Computing) with the package *maps* (v3.1.0) using the *mapdata* (v2.2-6) “worldHiresMapEnv” and the package *plotrix* (v3.6-2) for pie charts.

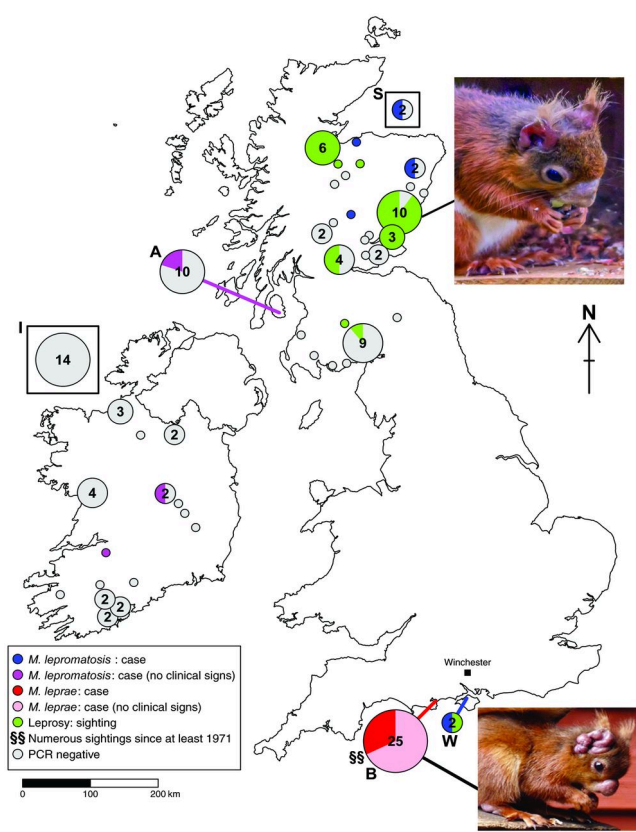


Fig. 2. Gross histopathological features of red squirrels with leprosy. (A) Both macroscopic and histological features of squirrels infected with either *M. lepromatosis* or *M. leprae* are similar. (B) Histological examination of tissue sections from infected squirrels using the Ridley-Jopling (RJ) classification following Ziehl Neelsen staining (Mag. x400). LL: lepromatous leprosy, BL: borderline lepromatous leprosy. (C) Summary of main macroscopic and microscopic findings from squirrels infected with *M. leprae* (n=8) or *M. lepromatosis* (n=4).

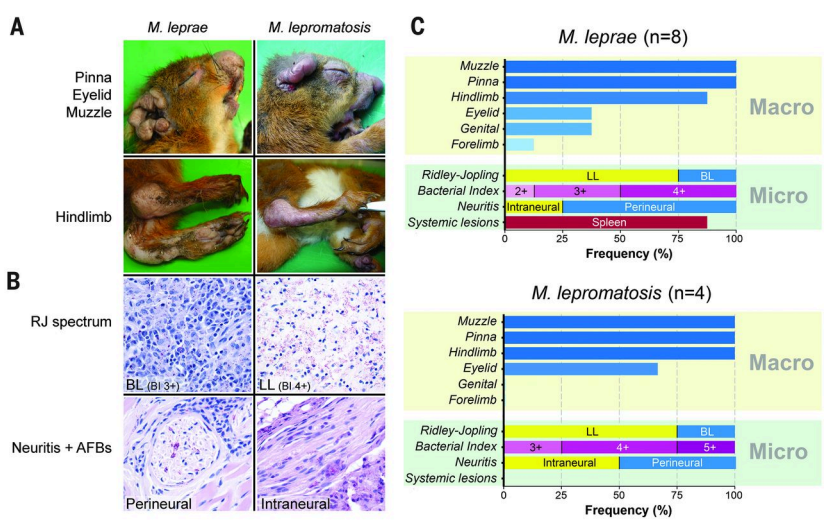


Fig. 3. Phylogeny of leprosy bacilli. (A) Bayesian phylogenetic tree representation of nine *M. lepromatosis* genome sequences obtained from squirrels (bold) or humans, upper and lower parts, respectively, calculated by BEAST 1.8.2 (24) using the mutation rate of *M. leprae* and inferred from 432 genome-wide variable positions. Squirrel sample prefixes: Ir, Ireland; Iow, Isle of Wight; with all others from Scotland. Both human strains were from Mexico. (B) Bayesian phylogenetic tree representation of *M. leprae* inferred from 498 genome-wide variable positions, calculated as in (A). Squirrel samples (bold): Brw denotes Brownsea Island cluster with red labeling indicating ancient strains for which radio-carbon dating information was available (3). For both trees, divergence time intervals are shown on each node in years before present, with the 95% HPD range in brackets. Posterior probabilities for each node are shown in grey.

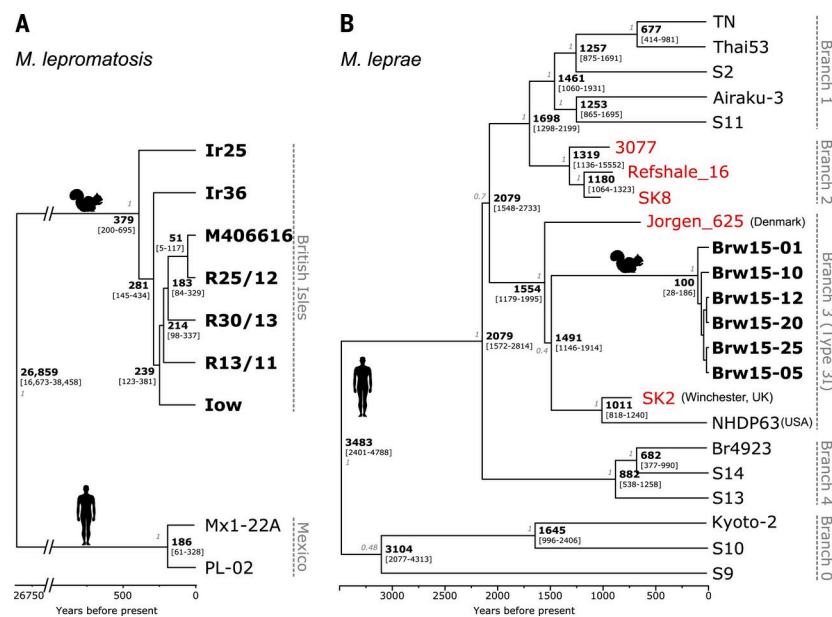
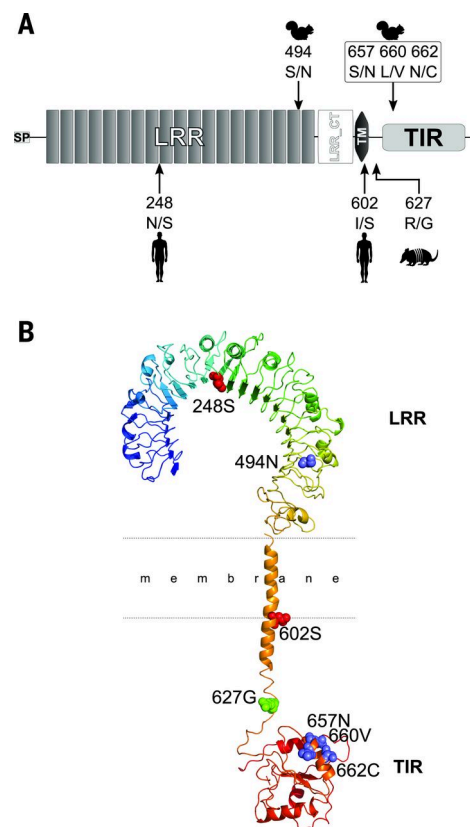


Fig. 4. Organization, structure and polymorphisms in TLR1 associated with leprosy in humans, armadillos and red squirrels.. (A) Schematic representation of TLR1 and its domains (drawn to scale). SP = Signal peptide, LRR = Leucine-rich repeats, LRR_CT = Leucine-rich repeat C-terminal, TM = transmembrane domain, TIR = Toll/interleukin-1 receptor. (B) Structural model of the red squirrel TLR1. Protein is colored in a rainbow spectrum from N-terminus (blue) to C-terminus (red).



344

345 **Supplementary Materials:**

346 Materials and Methods

347 Figures S1-S5

348 Tables S1-S14

349 References (31-51)

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